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PHYSIOLOGY OF STOMATA OF RUMEX PATIENTIA*

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INTRODUCTION.

The periodicity of transpiration cannot be satisfactorily explained until more is known about the exact behavior of stomata. The object of this paper is to record certain investigations of the conditions and changes occurring within the leaves and in the environment which are correlated with the high day and low night rates of transpiration. These investigations have been confined to a single species of plant, patience dock—(Rumex patientia L.) and the conclusions drawn apply to this plant, and perhaps to others.

Continuous records of environmental factors and of transpiration have been obtained, and synchronous observations of stomatal pores, starch content, sugar content, osmotic values, and H-ion concentrations of the guard cells have been made. The results are reported in the following pages.†

Patience dock was selected for this work because it has very large guard cells. The epidermis which is only slightly cutinized can be very easily removed from the large smooth leaves which are entirely free from epidermal hairs or appendages. The plants grow readily in the greenhouse or in the garden.

Stomata are present on both surfaces of the leaves. Numerous counts showed more per unit area near the margin than near the midrib of the blade and more near the tip than the base of the blade. On mature leaves they average 32 per square

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millimeter on the upper surface and 49 per square millimeter on the lower surface of the leaf. The guard cells which resemble those of most mesophytic leaves, are surrounded by three or four rather small cells corresponding to subsidiary cells which can be easily distinguished from the larger, more irregular epidermal cells.

The wide-open pores on mature leaves average 18μ wide by 28μ long. The guard cells average 15μ larger in each dimension. Verbena ciliata and Fouquieria splendens, which Lloyd (1908) used in his investigations, have average pore openings of 10μ by 15μ and 9μ by 18μ, respectively. Eckerson (1908) reported the largest found in her observations as occurring on the upper surface of wheat leaves (grass type) 7μ by 40μ and those of Chrysanthemum frutescens (the common type) 11μ by 35μ. Pool and McKay (1916) reported those of alfalfa 3μ by 8μ. The large size of the stomata of patience dock is of decided advantage in making measurements of pores and microchemical tests of guard cell contents.

Part I.

Diffusion of Water Vapor Through the Stomata.

I. Diffusion of water vapor through small openings.

It has been known for some time that the exchange of gases and the loss of water from leaves occurs mainly through the stomata. The effect of variations in size of the pores on the rate of these processes is not as thoroughly understood. These experiments are confined to the loss of water vapor through the stomata since it is much easier to measure. The same principles of diffusion apply to all gases or vapors.

Brown and Escombe (1900) came to the conclusion that the static diffusion of gases through small openings is proportional to their diameters rather than to their areas. Table I confirms Brown and Escombe's results and shows very clearly that the diffusion of water vapor through small openings of this size in a thin septum is more nearly proportional to the diameters than to the areas of the openings. Since diameters and circumferences of circular openings are proportional it follows that diffusion is also proportional to the circumferences. It is possible to make two holes in a septum with the same circumferences or perimeters but with quite different shapes and areas;

in fact this is the case with the openings between the guard cells. When the guard cells are very wide open the pores are almost circular, but become elliptical and finally narrow slits as the guard cells close. If no changes in perimeter occurred on closing as much water vapor could diffuse through the pores when almost closed as when wide open according to this line of reasoning (neglecting the thickness of the septum).

Experiments were performed to see if Brown and Escombe's diameter law held when the shape of the hole was changed, but the perimeters remained the same. Two holes, one elliptical

TABLE 1.

DIFFUSION OF WATER VAPOR THROUGH SMALL OPENINGS IN THIN CELLULOID SEPTA, UNDER UNIFORM CONDITIONS.

Diameter of Hole in mm.	Diffusion of Water Vapor in grams	Ratios Areas of Holes	Relative Rate of Diffusion of Water Vapor	Ratios Diameter of Holes
2.64	2.655	1.00	1.00	1.00
1.60	1.583	.37	.59	.61
.95	.928	.13	.35	.36
.81	.762	.09	.29	.31
.72	.672	.07	.25	.27
.65	.590	.06	.22	.25
.56	.492	.05	.18	.21
.48	.455	.03	.17	.18
.41	.393	.02	.15	.15
.35	.364	.01	.14	.13

and one circular, were made in thin sheets of celluloid (Eastman Kodak Films) and the diffusion of water vapor through them was measured. Table 2 gives the results of these experiments which were repeated many times. Although these results do not agree as closely as those which illustrate the "diameter law," they do show that diffusion through an elliptical and a circular opening is more nearly proportional to the perimeters than to the areas of the openings. The elliptical opening has an area of only 14% of the circular opening, but there was 83% as much water vapor passed through the elliptical opening as through the circular opening under exactly identical conditions of temperature, humidity, etc. Exact agreement between perimeters could not be obtained although the experiments were repeated many times with different sized openings and under different conditions. In every case the results obtained showed quite

close agreement between perimeters but no relation to the areas of the openings.

It should be noted that these results are not the same as those previously reported on this subject. Stefan (1882) on theoretical grounds reported that the evaporation from a circular surface was almost the same as from an elliptical surface of the same area. But these results indicate that evaporation from a circular surface would be more nearly equal to that from an elliptical surface having the same perimeter, not the same area. As far as could be found no one has ever tried to verify Stefan's deductions in their relation to diffusion through circular and elliptical openings in septa. Brown and Escombe (1900) verified Stefan's deductions concerning the relation of diffusion through circular openings in a thin septa and found very

TABLE 2.

DIFFUSION OF WATER VAPOR THROUGH ELLIPTICAL AND A CIRCULAR OPENING IN SEPTA. AVERAGE OF 5 DETERMINATIONS WITH THE SAME SEPTA.

Opening	Perimeter in mm.	Area in sq. mm.	Diffusion of Water Vapor in gym	Perimeter	Ratios Diffusion
Circle.....	7.40	4.22	.743	1.00	1.00
Ellipse.....	7.40	.59	.608	1.00	.83

close agreement between diameters and diffusion when the holes were smaller than 5 mm. in diameter. Thomas and Ferguson (1917) showed that for circular surfaces of 2 to 10 cm. in radii that the evaporation is proportional to the (radius^2) 1.5 when the vessels are full of water and not to the (radius^2) 2 as Stefan's theory demanded.

In calculating the absorption of CO_2 and the loss of water through the stomata Brown and Escombe (1900) assumed that Stefan's deductions were correct and considered that the elliptical stomatal openings were equal to a circular opening of the same area in their diffusion capacity. Renner (1910) followed Brown and Escombe's assumptions in his work. Lloyd (1908), Livingston and Estabrook (1912), and Loftfield (1921) also made the same assumptions in expressing the diffusion capacity of the stomata. Livingston and Estabrook (1912) introduced the formula $\sqrt{ab}$ to express the diffusion capacity of stomata, a and b being the major and minor semi-axes of

the elliptical opening. This formula, $\sqrt{ab}$, is the radius of a circle whose equal area is to that of the elliptical opening, and is derived from the formula for the area of an ellipse. But since the pores are not true ellipses and vary considerably from true ellipses especially when the stomata are almost closed the best representation of the diffusion capacity of the pores would be their average perimeters. This can be measured easily with a small flexible rule from camera lucida drawings or photographs of the pores.

In considering the diffusion of gasses through stomatal openings a "Perimeter Law," i. e., variations in the rate of diffusion in proportion to the perimeter of the openings would more nearly represent the actual diffusion capacity of the stomata than Brown and Escombe's "Diameter Law."

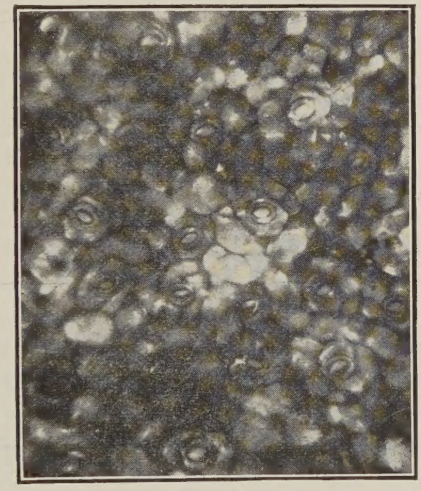

FIGURE 1.

Photograph of the surface of an uninjured leaf of patience dock showing the stomata in position. (About 90×.)

II. MEASUREMENTS OF PORE DIMENSIONS.

The study of the size of the pores of the leaf was made by the second method suggested by Lloyd (1913) of direct observation in position on the uninjured leaf. It is possible to see clearly and measure accurately or even photograph (see Figure 1) the stomata of patience dock because of their large size, with a lens having a 4 mm. working distance and a 12.5 eyepiece. An ordinary condenser and light from the northern sky, instead of

direct sunlight, was used; thus no injury to the leaf resulted from overheating. Careful checks were made and no injury to the leaf or change in the size of the pore resulted as reported by Loftfield (1921), even from repeated observations on the same place of a single leaf.

This method was chosen in preference to the absolute alcohol method also introduced by Lloyd (1908) and then later used by Loftfield (1921) because repeated checks always showed a narrower pore when the epidermis was removed and placed in absolute alcohol. Table 3 gives the results of a comparison of the two methods. Ten large pores were measured on the lower surface of the leaf and the epidermis from this same area was quickly removed and placed in absolute alcohol. Measurements were then made from the material in absolute alcohol.

TABLE 3.

COMPARISON OF SIZE OF PORES IN POSITION ON THE UNINJURED LEAF WITH THOSE ON CUT AND STRIPPED PIECES OF EPIDERMIS WHEN PLACED IN DISTILLED WATER AND IN ABSOLUTE ALCOHOL. AVERAGE OF 20 PORES.

Size of Pores	In Position on the Leaf	In Distilled Water		In Absolute Alcohol	
		Cut	Stripped	Cut	Stripped
in μ	12 x 33	3 x 33	14 x 29	3 x 29	7 x 28

A comparison of the results shows that caution must be used in the absolute alcohol method, because removing the epidermis from the leaf may change the size of the pores.

The average size of the stomata when they are wide open is 18μ by 28μ, and the maximum is 20μ by 36μ. They vary from 37 per square millimeter on the lower surface at the base to 60 per square millimeter at the tip and from 26 to 38 per square millimeter on the same places on the upper surface of the mature leaf. Younger smaller leaves show more per unit area then the older larger ones. The average is 49 on the lower surface and 32 on the upper surface of the leaves. Comparing the elliptical opening to a circle having the same perimeter and assuming that they are spaced about equal distance apart, 32 stomata 18μ by 28μ, which is the average size, would be 9.3 diameters apart and 49 per square millimeter would be 7.2 diameters apart. Thus the stomata average 7.2 to 9.3 diameters apart on the leaves. According to Brown and Escombe's

results, there is little or no interference in diffusion between stomata when they are 8 to 10 diameters apart. That is, at or beyond this distance apart each stoma functions as a single opening.

From these results we can conclude that in patience dock each stoma acts practically as an independent unit. Any formula which would express the relation of the size of the pores to the rate of water loss through them should be derived from the linear dimensions of the pore and not from the area, since as shown by Table 2 the diffusion through elliptical openings is more nearly proportional to the perimeter than to the area. Variations in the perimeter of the pore, therefore, would more nearly represent the relation of the size of the pores to water loss through them.

III. COMPARISON OF PORE DIMENSIONS.

A comparison of perimeter, area, and width of the pores during opening and closing and the normal daily variations in these dimensions was made by direct observation on the uninjured leaves. Measurement of camera lucida drawings of the pores at different degrees of opening were also made. Much data were obtained from these studies, and since space is limited a summary only is included here.

Figure 2 shows the perimeter and area of the average pore (18μ by 28μ) at different stages of opening. These results are actual measurements of areas with a planimeter and perimeter with a flexible rule of camera lucida drawings of the pores at different degrees of opening. An examination of these curves shows that the area of the pore is about proportional to the width of the opening, but in the case of perimeter this is quite different. When the pore is open only 10% or 1.8μ in width the perimeter is 60% of the maximum. Thus, according to the diffusion law as applied to perimeters, the amount of water vapor which could diffuse through the pore when it is 10% open is 60% of the maximum; but since actual experiments have shown only an average of 83% as much diffusion through an elliptical as through a circular opening of the same perimeter we must modify this accordingly; so from actual physical experiments about 50% (83% of 60) of the maximum amount could diffuse through the narrow slit as compared to the wide

elliptical opening. On closing, therefore, the rate of water loss through a stoma would decrease about 50% until the pore closed completely when the amount would be 0%.

IV. DAILY VARIATIONS IN PORE DIMENSIONS.

Figure 3 shows the width, area, and perimeter of the pore during a 24-hour day. Observations were made every hour as

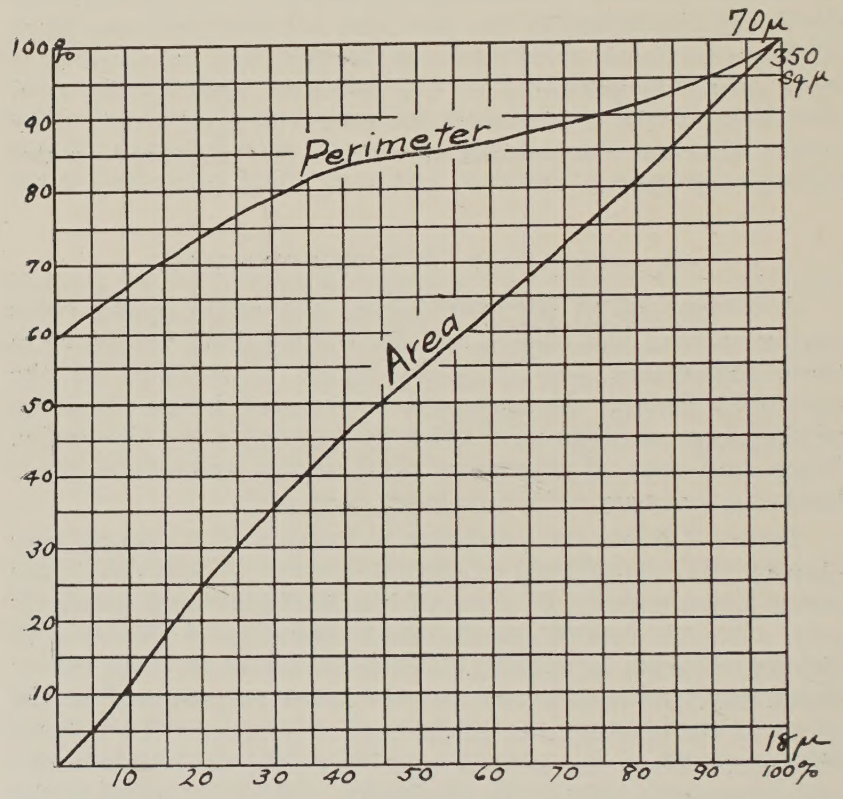

FIGURE 2.

Perimeter and Area of the average pore (18μ × 28μ) at different stages of opening.

long as the stomata were found open. Records were made throughout the night, but the stomata of patience dock were never found open at night, even in bright moonlight. This is the opposite of their behavior as reported by Loftfield (1921). The first indication of opening occurs shortly after daylight in the morning and many may be found open at sunrise on a

bright morning. On cloudy days opening is slower. Figure 3 shows a gradual increase in width of the pores until a maximum was reached at about noon and then a gradual decrease in width until they were completely closed at sundown. This is not the behavior that always occurs, however, for the degree of opening is influenced very much by environmental conditions, especially, humidity, the amount of water in the soil, and the temperature.

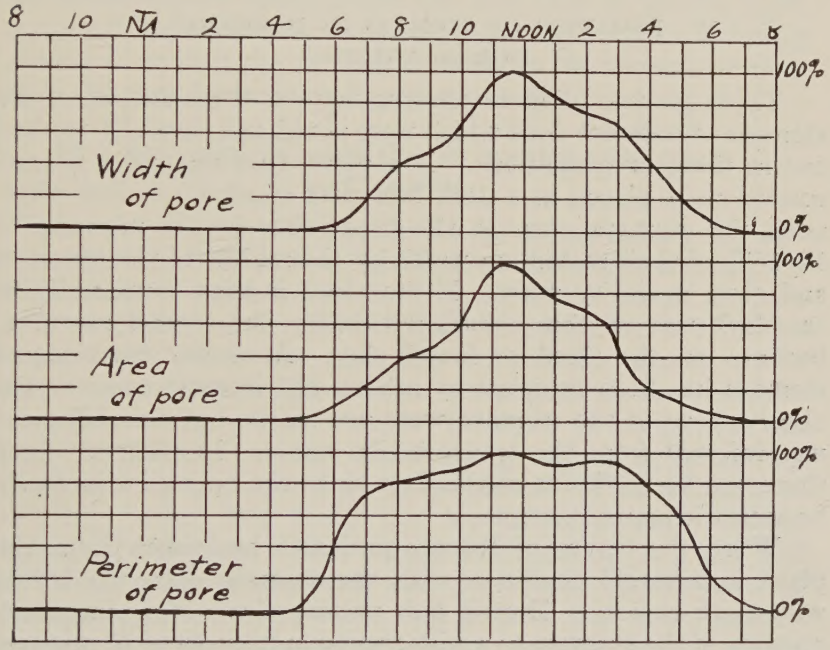

FIGURE 3.

Daily variations in the width, area, and perimeter of the average pore (18μ × 28μ).

On a bright clear warm morning maximum opening may be reached at 9 o'clock and if a favorable water balance is maintained throughout the day they may remain open until 5 o'clock in the afternoon. If the plant wilts slightly during the middle of the day closure results and opening may occur again in the late afternoon if wilting is not complete. If the plant is badly wilted they close and remain closed the rest of the day even in bright sunlight. Stomata on badly wilted plants which do not regain their turgidity at night do not open in the morning. No difference in the general behavior of the stomata on the two

surfaces of the leaf could be determined in all these observations. Loftfield (1921) reported midday closure of the stomata of patience dock and other plants; also night opening under certain conditions, especially lack of water. Although midday closure was very common, opening at night was never observed by the methods used in this work, even when the plants were not supplied with water until permanent wilting resulted.

V. BEHAVIOR OF STOMATA IN DARKNESS AND DURING WILTING.

There are two other interesting facts in the behavior of the stomata of patience dock which were noted and carefully worked out in these investigations. If a plant growing under normal conditions is placed in a dark box after sundown or late afternoon the stomata open at the usual time in the morning to 10–15% of their maximum width by the middle of the forenoon and close again by noon. If the plant is kept continually in the dark this rhythm occurs noticeably the second morning, but not on the third or fourth day. A similar behavior of stomata has been reported by others, but in most cases actual observations of the stomata were not made, but this behavior was inferred from the transpiration curve. In patience dock there can be no doubt of this fact since the opening can easily be seen with a microscope.

When the leaves of Rumex patientia are taken from the plant in a turgid condition with the stomata open the leaves wilt quite rapidly. During this wilting there is a temporary opening of the stomata to a wider value in from 10 to 15 minutes after the leaf is taken from the plant; and then there is a closure of the stomata on the wilted leaf. Wilting is noticeable a few minutes after the leaf is taken from the plant, but the stomata are not closed for 20–30 minutes. Darwin (1898) reported this behavior in a number of plants with which he worked. Laidlow and Knight (1916) by means of a recording porometer showed very clearly this behavior in Phasolus vulgaris. Lloyd (1908) could find no indications of this temporary opening in his work.

In patience dock this beahvior is very easy to observe because of the large guard cells. In position on the uninjured leaf 10 stomata averaged 10μ in width. Six minutes after the leaf was cut from the plant 10 stomata from the same place on the leaf averaged 17μ in width and 5 minutes later, 18.4μ. In 28 minutes after the leaf was cut from the plant all the stomata were closed.

If on the other hand the leaf is attached quickly to the water tap instead of allowing it to wilt, and water is forced into the leaf under a pressure of about 3 atmospheres the opposite behavior results; the stomata close immediately to about 10% of their maximum width. If the pressure is removed so that the intercellular spaces do not fill with water the stomata will return to their former opening and on wilting they will close. These observations substantiate the fact that in general light is the most important factor concerned in opening and closing of stomata but that the amount of water in the leaves also may modify the degree of opening very markedly.

VI. RELATION OF STOMATA TO WATER LOSS FROM THE LEAVES.

That the loss of water from the leaves occurs mainly through the stomata has been proven by numerous investigators; but the modifications of the rate due to variations in the size of the pore is not as clearly understood. That complete closure of the stomata stops water loss from the intercellular spaces of the leaf can not be doubted, but some investigators seem to doubt if complete closure ever occurs. In these investigations an attempt was made to solve these two problems as far as the water loss from patience dock is concerned. Two methods of measuring water loss from the leaves were used. Direct weighing of the plant which necessitated sealing in a pot and thus limiting the root system; and the cobalt chloride method of comparing the rate of water lost from a free water surface with that from the leaves of the plant. In this method plants growing in the soil can be used.

Figure 4 shows the hourly rates of water loss from the plant and evaporation from a porous cup under similar environmental conditions. These data were obtained by weighing a sealed potted plant and an atmometer at hour intervals throughout the day. The environmental factors were recorded by automatic recording instruments. The transpiration curve shows that there is a periodic water loss from the plant; a very low constant rate during the hours of darkness with a very high rate during the day. The curve of water loss from the porous cup is very similar to the transpiration curve and one might think at first that the periodicity of water loss from the plant is due simply to the increased "evaporating power" of the air. But a careful examination of the data shows that this is not true. When the

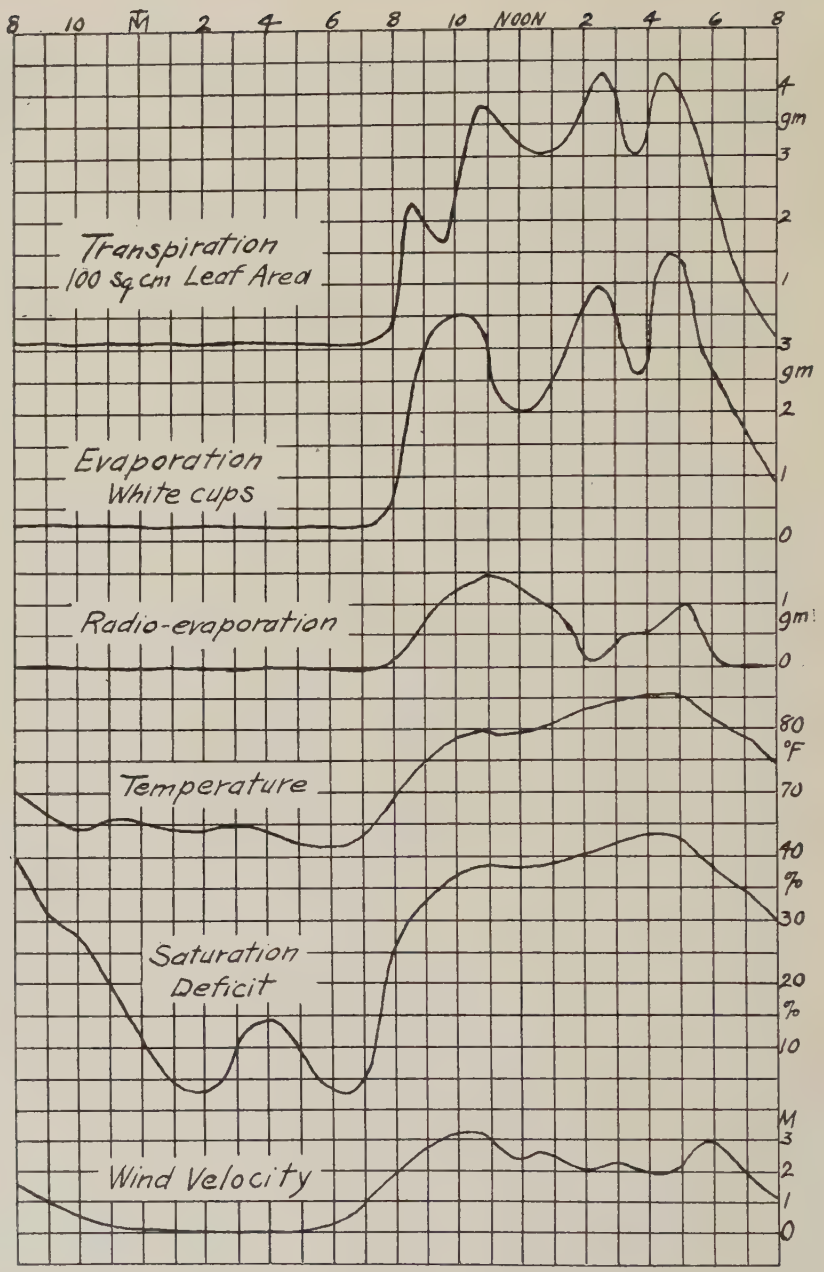

FIGURE 4.

Comparison between transpiration and environmental factors.

average hourly night rates of evaporation and transpiration are compared with the same values for the day it is seen that although the increase of day over night rate is considerable in each case the rate of water loss from the plant has increased many times more than the rate of evaporation. Table 4 shows a comparison of the data given in Figure 4 made in this way. The average day rate of transpiration was 51 times greater than the average night rate, while evaporation has increased only 17 times. Even in the case of blackend cups, which absorb heat energy to a greater degree than the green leaves and be at a temperature slightly higher than the air, the increase was only 19 times. Since the same environmental factors tend to increase both processes and are the same in each case, the conclusion is

TABLE 4.

AVERAGE DAY AND NIGHT RATES OF TRANSPIRATION AND EVAPORATION UNDER SIMILAR ENVIRONMENTAL CONDITIONS.

	Grams per Hour		Increase Day Rate Over Night Rate
	Average Day	Average Night	
Transpiration.....	2.81	.055	51 times
Evaporation:			
White cups.....	2.88	.163	17 times
Black cups.....	3.21	.163	19 times

obvious that some factor in the plant causes the added increase of day over night rate. When the data for Figure 4 was obtained the air was almost saturated at night with no wind and the following day was clear, hot, with quite variable wind velocities, thus presenting almost two extremes in environmental factors. A similar set of data was obtained in the winter in the greenhouse, where the environmental factors between day and night were more uniform. Wind was eliminated from the experiment in the greenhouse. The increase of day over night rate in this case is not as great. Transpiration in this experiment increased 9.7 times, while evaporation increased only 3 times. In both experiments transpiration has increased about three times as much as evaporation.

There are several possible causes of this periodicity of water loss from the leaves; variations in amount of water in the leaves, changes in the osmotic concentration of the mesophyll

cells, changes in H-ion concentration of the mesophyll cells, and opening and complete closure of the stomata, thus shutting off water loss entirely from the intercellular surface of the leaf.

The amount of water in the leaves was found by drying at 104° C. to constant weight leaves without the petioles or midribs in glass stoppered weighing bottles. The average amount of water in the leaves at night when they are turgid and crisp was 90.95%. There was no decrease in this amount until about 10:30 in the forenoon, when the amount had decreased to 89.3% in one of the experiments. There was a gradual decrease from that time until between 3–4 P. M., when the minimum of 88.8% was reached. Although the leaves were still erect, they were

TABLE 5.

OSMOTIC VALUE AND PH VALUE OF THE JUICE PRESSED FROM THE FROZEN LEAVES OF PATIENCE DOCK.

June 26	Osmotic Value in Atmo.	pH Value of the Juice
8 A. M.	9.26	3.9
10 A. M.	9.40	3.8
12 Noon	9.65	3.8
2 P. M.	9.95	3.8
4 P. M.	9.86	3.8
6 P. M.	9.39	3.8
8 P. M.	9.23	4.0

noticeably flaccid and not as crisp as at night, when they contain the maximum amount of water. The amount increased after 4 P. M. until at 9 P. M. the maximum amount was present again. Livingston and Brown (1912) reported a decrease in leaf water content of about 2% in the plants with which they worked. Knight (1922) also reported about the same decrease. In neither case were the leaves wilted. Under ordinary conditions there is a daily variation of about 2% in leaf water content of the leaves of patience dock.

Table 5 gives the osmotic pressure in atmospheres and the pH value of the sap pressed from the leaves (without midribs) at 2-hour intervals during the day. The leaves were frozen, the sap pressed out and the depression of the freezing point determined with a Beckman thermometer. The pH values were determined by the colorometric method of Clark (1920). Freezing caused no change in pH value of the sap as checks

with frozen and unfrozen tissue showed the same value. Determinations in the depression of the freezing point at night showed no further decrease in the values. This table shows that there is an increase in the osmotic value of the sap during the day reaching a maximum at about the same time the leaf water content reaches a minimum. The H-ion concentration fluctuated between pH 3.8–4.0, but with no consistent variations which would indicate that there was a daily change in the pH value. These variations are within the limit of error of the method used.

The decrease in leaf water content and increase in osmotic concentration during the day would tend to decrease the rate of water loss from the leaves. But these factors appear to have no very decided effect on the rate of water loss because it is greatest when these factors would tend to reduce the amount. Changes in acidity alter the hydration of colloids, but no consistent change occurs in the leaves of patience dock, so the only internal condition left to consider which would cause such a large increase of day over night rate is the opening and complete closure of the stomata.

Observations on the stomata of the uninjured leaves showed that they were always closed at night. No exception to this rule was found. They were always found open in the daytime if the leaves had not wilted. On wilted leaves they were often found closed or almost closed, depending on how long wilting had been evident. But the objection has been raised that closure which appears complete under a microscope does not mean hermetically sealing so that no water vapor can escape through them.

An experiment was performed which shows that the stomata of patience dock are closed tight at night so that water vapor does not diffuse through them. Two similar plants were taken, one placed in a dark box and the other on a table beside it. The following day the time required for standard cobalt chloride paper to change from blue to pink on similar leaves of each plant was determined. Cobalt chloride paper from the same sheet was taken for the test and both plants were at the same temperature so the results are comparable. An examination of the plants just before the tests were made showed that the stomata on the plant in the light were open, while those in the dark were closed. The paper changed to red in one minute and 40 seconds on the lower surface of the leaf with open stomata,

while 75 to 80 minutes were required to produce the same color change on the leaf with closed stomata. Assuming that the rate of water loss is proportional to the time of color change, water is lost from the leaf with open stomata 45 times as fast as when the stomata are closed. Since according to the "diameter law" modified to include elliptical openings, any preceptible opening between the guard cells would allow 50% of the maximum rate of water loss it is safe to conclude that no water vapor is lost through the stomata when they are closed. The change in the cobalt chloride paper on the leaf with closed stomata represents the rate of water loss from the epidermis,

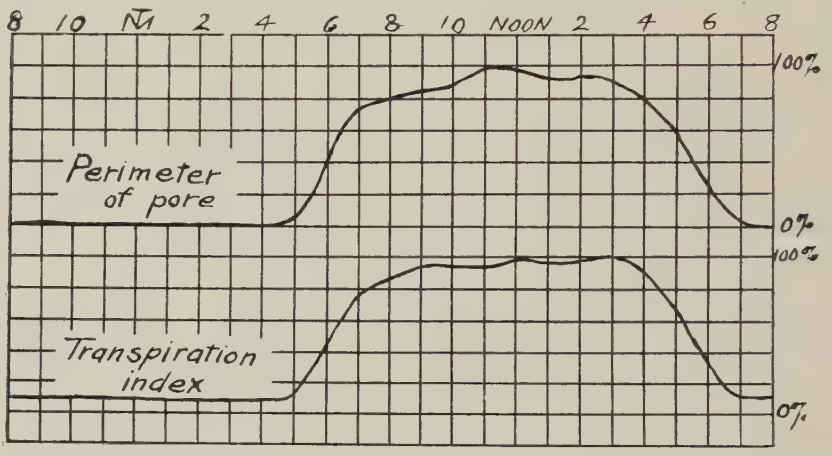

FIGURE 5.
Comparison between transpihation index and perimeter of the stomata.

since very similar results are obtained from test on leaves with stomata only on one surface. In Vinca where the stomata occur only on the lower surface, the same cobalt chloride paper turned red in 2 minutes when the stomata were open, but 60 to 70 minutes were required for the same change under the same conditions on the upper surface of the leaf.

The most important cause of the periodicity of water loss from the leaves of patience dock is the opening and complete closure of the stomata. At night when the stomata are closed, there is a very low constant rate because the environmental factors on which this epidermal water loss depends are very constant. But during the day when the stomata are open, the rate is very high and quite variable from time to time. This is

due to the fact that the factors on which the rate depends are many and are apt to vary considerable. Environmental factors are usually more variable during the day than at night. The internal factors are also quite variable and some tend to increase the rate, while others tend to decrease it and no single factors can be found on which the rate depends entirely. An examination of the general shape of the curve of water loss during the day, however, will show that it more nearly corresponds in its main outline to the curve for average perimeter of the stomata.

When the curve of transpiration index determined by the cobalt chloride method is compared with the curve for perimeter of the stomata there is still closer agreement between them. Figure 5 gives the curve for transpiration index and perimeter of the stomata. These data are the average of a number of determinations of the time of color change of standard cobalt chloride at each hour and for the same place on each leaf along with the average size of the pores on those same areas of the leaves. The curve of transpiration index and perimeter of the stomata are almost the same because in this method of measuring the relative water loss all environmental factors are eliminated. Thus the curve of the transpiration index would be more nearly a curve of the function of the stomata. This curve does not indicate the actual rate of water loss from a plant under ordinary conditions. When the cobalt chloride paper is placed on the leaf air currents are absent and there is a layer of dry air next to the leaf, since the cobalt chloride paper is perfectly dry when used. It is equivalent to measuring water loss from a plant surrounded by perfectly dry still air.

These variations in the day rate of water loss as shown by Figure 4 are due mainly to differences in temperature, humidity, wind velocity, (caused by cloudiness), and leaf water content, the main shape of the curve being due to the opening and closing of the stomata.

VII. SUMMARY.

Patience dock was selected for this study of the physiology of the stomata because of the very large guard cells. The leaves are large and smooth and the plant grows well under ordinary conditions. The epidermis can be easily removed so that the guard cells can be studied under varying conditions.

Brown and Escombe's laws of diffusion of gases through small openings were checked and were further shown to apply to elliptical openings.

Checks on the methods of measuring the dimensions of the pores were made and the method of measuring the pores on the uninjured leaf was chosen because of the variations shown when the epidermis was removed from the leaf. A comparison of the area, perimeter, and width of the pore during opening and closure was made. The width of the pore is the best means of expressing the degree of opening of the stomata.

When the dimensions of the pores were measured during the day and under different environmental conditions considerable variations were found. It was necessary to average a number of measurements in order to have comparable results. A rhythm of the opening of the stomata was observed in darkness after a day in light and a temporary increase in the width of the pore was noted when permanent wilting began.

In a study of the relation of stomata to transpiration the effect of complete closure of the stomata was determined and an attempt was made to correlate the variation in the pore dimensions with the rate of transpiration from the leaves.

Part II.

Physiology of the Guard Cells.

I. Osmotic relations of the guard cells.

Since the time of Von Mohl, 1856, the opening and closing of the stomata has been explained by changes in turgor of the guard cells; but few investigators have made direct measurements of this turgor. Iljin (1914) was the first to attempt a measure of the pressure within the guard cells and the epidermal cells. He reported the average osmotic value of the guard cells to be 90–100 atmospheres when open, while the epidermal and mesophyll cells were about 20 atmospheres. His determinations were made by the plasmolytic method using KNO_3 in different concentrations as a plasmolysing solution. Wiggins (1921) using $CaCl_2$ as a plasmolysing solution, determined the osmotic value of the guard cells of Zebrina pendula, Cyclamen, Iresine, and sugar beet. He found a higher pressure in the guard cells than the surrounding cells when the stomata were open, but his

results were very lower than those obtained by Iljin (1914), the difference between guard cells and epidermal cells averaging about 13 atmospheres.

The method of determining the osmotic value of the guard cells of patience dock in this work was the usual plasmolytic method, taking as the osmotic value of the cells that concentration in which the cell contents showed the first indications of shrinkage away from the cell wall. Two methods of applying the different concentrations of the plasmolysing solution were used. In the first method small pieces of the tissue were placed in about 1 cc. of the different concentrations in a small vial and examined after 2–5 minutes in a drop of that solution on a slide under a microscope. In the second method a small piece of tissue was placed across a narrow groove in a slide, covered with a cover glass and the different concentrations of the plasmolysing solutions were drawn through under the cover glass with a filter pump. From 1 to 2 cc. of the different solutions were used and allowed to remain in contact with the tissue from 1–2 minutes or until no further change occurred in any of the cells. The two methods gave very similar results in all checks. The last method has the advantage of requiring less material and of permitting continuous observations on a single group of cells. The uniformity of the results obtained by these two procedures indicated that the method is as accurate as any other of the plasmolytic methods. The results are usually too high because of some cell shrinkage before the protoplasm pulls away from the cell walls. Knudson and Ginsburg (1921) showed that if the tissue is properly frozen and high pressures are used in extracting the juice that the freezing point method and the plasmolytic method gave very similar results.

All solutions used were standardized by the depression of the freezing point method and the results are expressed in atmospheres. Glucose, cane, sugar, $CaCl_2$ and KNO_3 were used in order to eliminate, if possible, differences in permeability of the guard cells. The osmotic value of .1M, .2M, etc., concentrations of the solutions were determined and a curve of the values plotted from which pressures of any desired concentration could be read. Dilutions of the stock solutions were made having values of from 1.2 to 2.5 atmospheres, depending on the substance used. Records of pore dimensions and

environmental factors were obtained for each period that osmotic values were made.

Such wide ranges of variations in the osmotic values were found that it was necessary to observe from 30 to 40 guard cells

TABLE 6.

OSMOTIC VALUE IN ATMOSPHERES OF THE GUARD CELLS, SUBSIDIARY CELLS, AND EPIDERMAL CELLS, USING DIFFERENT PLASMOLYSING SOLUTIONS.

Time August 2, 1921		8:30 A.M.	10:30 A.M.	1:30 P.M.	3:30 P.M.	7:30 P.M.	
Stomata		3x30	6x31	12x34	9x31	0x0	Microns
Guard Cells:	Maximum	19.1	17.8	23.0	21.4	14.4	Cane sugar
	Average	16.6	16.6	21.0	20.2	13.2	
Subsidiary cells		16.6	16.6	16.6	17.8	15.5	
Epidermal cells		14.4	14.4	14.4	14.4	13.2	
Guard Cells:	Maximum	16.2	16.2	19.6	17.8	14.0	Glucose
	Average	15.1	15.5	17.3	16.6	12.9	
Subsidiary Cells		16.2	15.1	16.2	16.6	15.1	
Epidermal Cells		14.0	14.0	14.0	15.1	12.9	
Guard Cells:	Maximum	18.4	18.4	21.9	20.0	12.4	$CaCl_2$
	Average	17.5	17.5	18.4	18.4	12.4	
Subsidiary Cells		14.8	15.7	15.7	15.7	13.0	
Epidermal Cells		13.0	14.2	13.0	14.2	12.4	
Guard Cells:	Maximum	19.8	?	?	?	12.8	
	Average	15.1	?	?	?	11.8	KNO_3
Subsidiary Cells		14.0	15.1	16.2	15.1	10.4	
Epidermal Cells		10.4	10.4	11.8	11.8	10.4	

to obtain average results which were comparable. The average values of the subsidiary cells, and epidermal cells were noted and the average and maximum values of the guard cells were determined. Because of the fact that pieces of the epidermis were cut and placed in water before starting the determinations it was impossible to measure the size of the openings of individ-

ual stomata and their osmotic value. The pore dimensions are the average values from the same areas of the leaf before the epidermis was removed.

Table 6 shows the osmotic values in atmospheres of the guard cells, subsidiary cells, and epidermal cells, with average pore dimensions from the upper epidermis of leaves of patience dock. Marked differences between some of the values in this table are shown. These differences are due, no doubt, to the difficulties encountered such as distinguishing between the effect of a solution of 15 atmospheres and one of 16.5 atmospheres, variation in individual leaves and plants due to age, water supply, light, etc., since it is impossible to use the same piece of epidermis for more than one determination.

The results shown when glucose, cane sugar, and $CaCl_2$ are used are quite similar, but those obtained from KNO_3 are not consistent. The highest concentration of KNO_3 used, 27 atmospheres, failed to show any indications of plasmolysis when the stomata were wide open. A test of the peremability of the guard cells to the different substances showed that when open the guard cells of patience dock were readily permeability to KNO_3. This suggests an explanation of the high values reported by Iljin (1914) when only KNO_3 was used. These results agree quite closely with those reported by Wiggins (1921). The osmotic value of the epidermal cells are quite constant throughout the day, the variation being due to differences in material or experimental errors rather than real differences in concentration. The average value is about 13 atmospheres. The subsidiary cells show similar variations but are always higher, averaging about 15 atmospheres. The guard cells show values when they are closed below the subsidiary and about equal to the epidermal cells, but much higher values when they are opened. The maximum value was as high as 23 atmospheres when fully opened, but averaged 19 atmospheres. Values as high as 27 atmospheres were noted in some of the trials. The osmotic values when the stomata were closed at night were carefully checked and the results showed that the guard cells were 1 to 2 atmospheres lower than the subsidiary cells. Thus the guard cells were actually pressed together at night when they are closed.

Figure 6 shows the results of a similar experiment on the guard cells and the surrounding cells of patience dock using only cane sugar as the plasmolysing solutions. The results

are expressed graphically and show the average pore dimensions from the same leaf that the pieces of epidermis for the tests were taken. The same general results as described in Table 6 were obtained. Many other measurements were made on the osmotic values of the guard cells, but space does not permit the presentation of all these data. These data showed that there was no appreciable difference between the osmotic value of guard cells on either surface of the leaf, and that the average

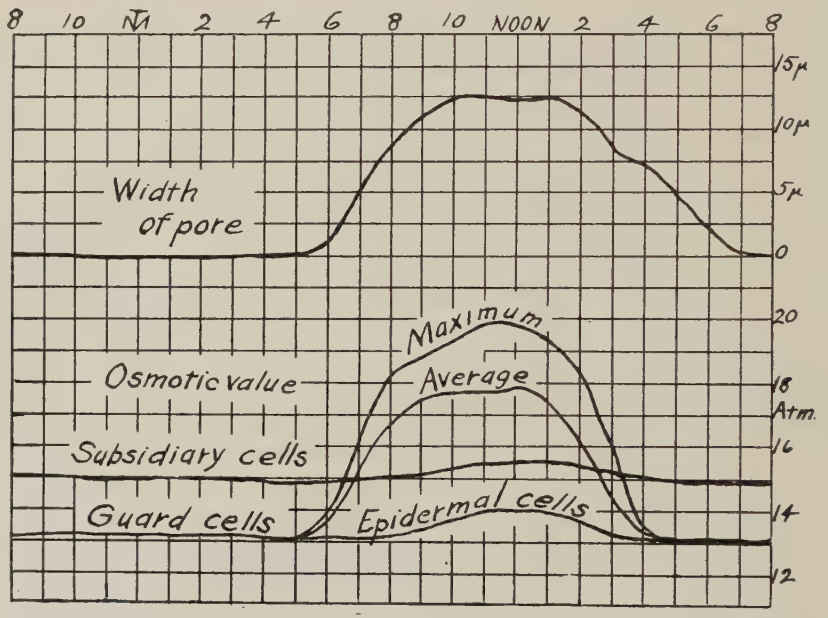

FIGURE 6.

Comparison between the osmotic value of the guard cells and the width of the stomata.

pore dimensions do not always correspond very closely with the average osmotic values of the guard cells. The midday closure reported by Loftfield (1921) was noticed in many cases, but no change in osmotic values could be found that was correlated with this closure unless the closure was complete, as in badly wilted leaves. There was no increase in osmotic pressure in the guard cells corresponding to the temporary opening just before wilting, but when the stomata closed on permanent wilting there was a decrease in osmotic value of the guard cells.

As is shown in Table 3, there is a partial closure of the stomata when sections of the epidermis and mesophyll are cut and placed in distilled water, but when the epidermis is stripped and placed in water the guard cells open wide. When these cut sections with the stomata partly closed are subjected to successively stronger plasmolysing solutions and the guard cells carefully observed, it is seen that the guard cells gradually open as the solution approaches the osmotic value of the epidermal cells; then as stronger solutions are added the guard cells close, and finally are plasmolysed. This behavior as well as midday closure and temporary opening on wilting, seems to be due to turgor changes in the surrounding cells as no corresponding changes in osmotic values of the guard cells were found which accounted for them.

It will be noted from Table 5 that the osmotic value of the mesophyll cells determined by the depression of the freezing point of frozen expressed juice was about 2–3 atmospheres lower than the values of the epidermal cells obtained by plasmolysis. It was impossible to see plasmolysis in the mesophyll cells in those sections used for the determination of guard cells. This would have been very desirable for a more accurate comparison. Iljin (1914) reported the mesophyll cells and the epidermal cells to have similar values. The difference in these results is probably due to the difference in methods of determination, since no powerful press was used to extract the juice from the frozen leaves and thus the values would be somewhat lower than those determined by plasmolysis. It was found that the osmotic values of all the cells of the different tissues of the leaf were from 2–3 atmospheres lower when the plants were growing in the greenhouse during the winter than when the plants were in the soil out of doors.

In conclusion it can be said that the increase and decrease in the osmotic value of the guard cells is the main cause of their opening and closing, but that osmotic pressure alone does not account for all their movements or changes in dimensions.

II. STARCH AND SUGAR CONTENT OF THE GUARD CELLS.

Darwin (1898) reported a change in the starch contents of the plastids of the guard cells during opening of the stomata. Lloyd (1908) proved that starch disappears from the plastids of the guard cells of Verbena ciliata and Fouqueria splendens

when they open. Iljin (1914) and Loftfield (1921) and Weber (1924) also reported the same observations. In patience dock the same change in starch content of the guard cells occurs.

In spite of the fact that Lloyd (1908) showed very clearly that the starch content of the guard cells decreased when the stomata open and thus their opening was not due to photosynthesis since in photosynthesis starch usually accumulates in plastids, many people still explain the behavior of the guard cells on this basis. The reason for this is due to their misconception about the plastids of the guard cells, which on casual examination appear to be chloroplasts. But a careful examination and consideration shows that they are quite different than the chloroplasts of the mesophyll. In patience dock the chloroplasts of the leaf are 5–6μ in diameter, appear as single structures and do not vary in size with their starch content. The plastids of the guard cells have at the maximum size a diameter of only 4μ, appear as compound structures, and vary in size with their starch content. The starch in both the plastids of the guard cells and the mesophyll cells does not show a cross with polarized light, thus indicating a colloidal form similar to starch paste rather than starch grains. Chlorophyll develops in the chloroplasts when exposed to light only, while the green color of the guard cells is shown under any conditions. The green color in the plastids of the guard cells does not give the micro-chemical tests for chlorophyll, but there is no conclusive proof that it is not chlorophyll because of the extreme difficulty of making the test on such small bodies. The green color of the plastids of the guard cells does not disappear in continued darkness while that in the chloroplasts does. The most striking difference, however, is in the occurrence of starch in the two structures. Starch is found in chloroplasts only in light and soon disappears in continued darkness. Some leaves as banana, lettuce, and onion, and probably many more, never show starch in the chloroplasts at all. Starch occurs in the guard cells of all plants examined under any condition where the guard cells are completely formed even in plants grown from seed in complete darkness, in white areas of variegated leaves, white shoots of plants, albino corn, etc. Onion is the only plant examined which did not show starch in the guard cells. Thus it can be easily seen that the plastids of the guard cells are different structurally, physiologically, and genetically, from the chloroplasts of the mesophyll cells.

The iodine method was used in measuring the starch content of the guard cells in this work. The amount of starch present by this method cannot be expressed in percentages because the test shows only a difference in the density of the blue color. Very slight differences, however, can be detected if two pieces of epidermis are mounted side by side after staining with iodine so that half the field of the microscope will be occupied by each piece. The results of these tests are expressed in four estimates; no change, very slight change, moderate change, and very striking change. They refer to an increase or a decrease in the

TABLE 7.

STARCH CONTENT, OSMOTIC VALUE, AND PORE DIMENSIONS OF THE GUARD CELLS FROM THE LOWER EPIDERMIS OF PATIENCE DOCK.

Time and Date	Pore Opening μ	Osmotic Value Atmo.	Starch Content
Aug. 4.			
7:30 A. M.	9 x 31	16.6	Starch present
9:00 A. M.	11 x 32	17.5	Moderate decrease
11:15 A. M.	5 x 30	15.7	Slight increase
12:45 P. M.	3 x 29	13.9	No change
4:00 P. M.	6 x 29	13.9	No change
7:30 P. M.	0 x 0	12.2	Slight increase
Aug. 5.			
8:00 A. M.	5 x 30	14.8	Moderate decrease
9:00 A. M.	9 x 31	15.7	Moderate decrease
10:00 A. M.	7 x 30	15.7	No change
12:00 Noon	6 x 28	16.0	Slight increase
2:00 P. M.	16 x 34	16.6	No change
4:00 P. M.	13 x 32	14.8	Silght increase
6:00 P. M.	0 x 0	12.2	Slight increase

starch content of a given specimen when compared with another specimen taken at some other time. Specimens were obtained for every hour of the day, kept in alcohol, which removed the chlorophyll and stained with iodine when examined. Table 7 gives the starch changes of the guard cells from the lower epidermis of patience dock together with the pore dimensions and osmotic values ($CaCl_2$ used as plasmolysing solution).

This table shows that there is a decrease in the starch content of the guard cells in the early forenoon which is correlated with the rise in osmotic values and increase in pore dimensions. On August 5 there occurred midday closure (partial) but there was no corresponding decrease in osmotic value or increase in starch content.

Lloyd (1908) pointed out that this fluctuation in starch content in the guard cells is just the opposite of that which occurs in the mesophyll cells of the leaf. An experiment extending over parts of two days was performed to show this variation. The width of the pore opening, starch content of the mesophyll cells and guard cells were determined by the methods previously described and are tabulated in Table 8. Starch increase in the mesophyll cells in the forenoon and reaches a maximum later in the day. But in the guard cells the starch content decreases in the forenoon when the mesophyll cells show an increase.

TABLE 8.

COMPARISON OF STARCH CONTENT OF THE GUARD CELLS AND MESOPHYLL CELLS OF PATIENCE DOCK.

Time and Date	Pore Width μ	Starch Content Guard Cells	Starch Content Mesophyll
March 13, '22			
7:00 A. M.	2	Starch present	Starch present
9:00 A. M.	11	Moderate decrease	Slight increase
11:00 A. M.	16	Moderate decrease	Silght increase
1:00 P. M.	17	Slight increase	Slight increase
3:00 P. M.	11	No change	Slight increase
5:00 P. M.	1	Moderate increase	No change
7:00 P. M.	0	Slight increase	Slight decrease
9:00 P. M.	0	No change	Slight decrease
March 14, '22			
7:30 A. M.	2	Moderate decrease	Decided decrease
9:00 A. M.	6	Moderate decrease	No change
11:00 A. M.	9	No change	Slight increase

The minimum occurs sometime before noon as Lloyd (1908) has shown in his work. There are slight fluctuations during the rest of the day with an increase again at night to the same amount as was present very early in the morning. No changes in the starch content of the guard cells during the hours of darkness could be detected. In the mesophyll cells, however, there is a gradual decrease during the night. Young rapidly growing leaves contain no starch by morning.

The decrease in starch content of the guard cells in the morning indicates that the rise in osmotic value is not due to photosynthesis. This is further proved by the behavior of guard cells in CO_2 free air. Lloyd (1908) found normal behavior of stomata in CO_2 free air. Patience dock showed normal opening and closure for four days in CO_2 free air. After the first day no

starch was found in the mesophyll and as the plant showed signs of starving the fourth day, the experiment was ended.

None of the investigators who studied starch fluctuations in the guard cells have attempted to show that sugars were present. They have assumed that if starch disappears sugar increases because of the usual reciprocal relation between the two and because the osmotic value rises when the starch content decreases. Micro-chemical tests for the presence of reducing sugars in the guard cells of Rumex patientia were made as follows: a small quantity of copper tartrate was mixed with 15% NaOH and a drop of this mixture placed on a slide, a piece of epidermis was placed in this mixture, a cover glass was added, and the slide was heated at 96° C. for 5 minutes. Equal sized pieces of epidermis were used and the same concentration of Copper solution was used, (mixed fresh before each test), so that the results are comparable. The sugar content of the guard cells was determined by estimating the number of copper oxide crystals formed which under identical test conditions showed from 2 to 2½ times as many when the guard cells were open as when they were closed.

We can conclude so far from this study of the physiology of the guard cells that the principal cause of the opening and closing of the guard cells is a change of the starch in the plastids of the guard cells to sugar which raises their osmotic value.

III. EFFECT OF CHANGES IN ACIDITY ON THE STARCH AND SUGAR CONTENT OF THE GUARD CELLS.

After the foregoing conclusions were obtained investigations were started to attempt to find out the cause of the change from starch to sugar in the guard cells. The reaction seems to be a reversible one in which the total amount of carbohydrate material does not vary, but is simply changed from one form to another under certain conditions. Since such changes in plants appear to be activated by enzymes the explanation of the change would be some condition which favors the action of the enzymes. The synthetic action of enzymes is inferred from the occurrence of compounds in the plant and little is known of the conditions under which they occur. But the hydrolytic action is easily demonstrated and much is known of the conditions favorable for this reaction. The H-ion concentration of the medium is important in the hydrolytic action of enzymes; there is an

optimum, a maximum, and a minimum value at which the reaction occurs.

All attempts to isolate enzymes from the guard cells failed because it was impossible to collect enough material to give a solution of sufficient concentration for a study of their reactions. But observations on the guard cells in different plasmolysing and buffer solutions furnished an explanation of the conditions favorable for the action of these enzymes. When the acidity of the guard cells was determined colorometrically by the use of Clark's indicators, it was found that the guard cells were slightly more alkaline with Bromphenol blue when they were opened than when they were closed. But it was impossible to determine the pH values because of no accurate means of color comparison under the microscope.

Attempts were made to find a buffer solution in which the guard cells would remain alive so that the effect of different H-ion concentrations could be studied. The phthalate buffers of Clark (1920) proved very toxic to the guard cells; the protoplasm appeared to be precipitated or coagulated when the guard cells were placed in this solution for an hour or more. Solutions of potassium oxalate and potassium phosphate were not as toxic as the phthalate solution, but it was difficult to make a balanced solution from these of a required H-ion concentration. Some results were obtained with these solutions, however, and will be mentioned later. The best results were obtained by using the juice from the leaves of the plant, patience dock, as a buffer solution. The juice was pressed from the leaves and dialysed with an equal volume of distilled water. This produced a clear liquid of pH value 3.8–3.9 and osmotic value of about 4 atmospheres. Guard cells appeared normal after 36 hours in this solution, no coagulation or precipitation of the protoplasm resulted. The pH value of this juice was adjusted to any desired value by adding small quantities of dilute HCL or NaOH.

When the guard cells were closed and were placed in this juice which was adjusted to pH values of 3.6–3.8–4.0, etc., to 5.0, they remained closed in the pH 3.6–3.8–4.0 solution, also in the pH 4.6–4.8–5.0 solution, but opened after 2 hours in the solution of pH 4.2–4.4. When the opened guard cells were placed in a series of these solutions they closed in all except the pH 4.2–4.4 after 2 hours or more. These tests were repeated many times and similar results were obtained each time. The

pH value of each solution was tested before and after it was used.

Similar experiments were conducted using phosphate buffers, oxalate buffers and various nutrient solution all adjusted as near as possible to the same series of pH values. Similar results were obtained in all the tests, but they were not as consistent and the guard cells did not open as wide or remain opened as long as in the dialysed juice. The optimum opening, however, occurred at about the same place in all cases; pH 4.2–4.4.

Determinations of the osmotic value of the guard cells and their starch and sugar content showed that when this opening occurred in the dialysed juice there was an increase in osmotic value and sugar content and a decrease in starch content. When closing occurred the opposite results were observed. Since this opening and closing of the stomata in the dialysed juice appeared to be just like that which occurs normally on the uninjured leaf the theory is advanced that changes in acidity are the cause of the starch to sugar changes in the guard cells.

Since these experiments were completed Weber (1923) has published his results on the effect of different ions on the guard cells. He found that Na- and K-ions favored the change of starch to sugar, while Ca-ions favor the change of sugar to starch. While determining the osmotic value of the guard cells of patience dock it was noticed that the starch content of guard cells which were plasmolysed several times with $CaCl_2$ increased considerably. But the same thing happened when cane sugar or glucose were used as the plasmolysing solutions, so it did not seem to be due to the Ca-ion. The concentration of the salt ion in the dialysed juice was not changed materially by the addition of small quantities of HCL or NaOH as were required to produce the desired change. The only factor which is changed in the series of buffers of the dialysed juice is the H-ion concentration, and as the guard cells open in these different concentrations it appears to be due to the change in H-ion concentration.

There is some further evidence to support this conclusion. The effect of acid and alkaline atmospheres on the guard cells was determined by the methods used by Small (1920) in the geotropic response of roots. Leaves with the stomata opened were placed in containers with varying amounts of dilute ammonia and acetic acid and the guard cells were studied. Opening to 50% of maximum occurred after 3–5 hours in ammonia vapor in darkness, but no opening occurred in acetic

acid vapor. Closure of opened stomata occurred in acid atmosphere in light, but not in alkaline atmosphere. Since it was necessary to enclose the leaf in a tight thick glass chamber it was not conclusive proof whether acid atmosphere or lack of light caused the closure. Over heating occurred if the containers were placed in full sunlight. These observations seems to further indicate that changes in acidity of the guard cells are the cause of the sugar to starch, or starch to sugar changes which result in their opening or closing.

IV. PROBABLE CAUSE OF THE CHANGES IN ACIDITY IN THE GUARD CELLS.

Normal opening and closing of the stomata does not occur except in light, although all other conditions may be made as favorable as possible. Light is the primary cause of opening and closing, but is not the only cause as is shown by the behavior of stomata in light during wilting and the failure of stomata to open on wilted plants in the morning. Light, therefore, can be said to cause a decrease in the acidity of the guard cells and in darkness the acidity increases again. This fact is shown by the colorometric tests on the guard cells, which show a more alkaline color with Bromphenol blue when opened than when closed; also from the behavior of guard cells in buffer solution of dialysed juice and in alkaline atmospheres. This decrease in acidity as indicated from the behavior of the guard cells in the dialysed juice is most likely from about pH 3.8–3.9, the normal acidity of the leaf juice, to about pH 4.2–4.4 in the morning and reversed in the evening. Whether there is a similar change during wilting can not be determined by these methods. The temporary opening at the beginning of wilting seems to be due to turgor changes of the surrounding leaf tissues. No change in osmotic value or starch to sugar changes were found accompanying this temporary opening, but on permanent wilting the osmotic value and starch content do change considerably.

The results shown in Table 5 on the pH values of the leaf juice indicate that there is no marked change in the acidity of the leaf in light or in darkness as apparently occurs in the guard cells. Acids accumulate at night in certain cacti, but titration tests showed no such change in patience dock. There is some evidence that light may change the acidity of the epidermal cells. When the clear juice from the leaves is titrated

with Na OH a reddish color appears at about pH 5.0–5.2. It is therefore seen that the juice contains a natural indicator.

When leaves which have been growing in reduced light are placed in strong sunlight a reddish color develops in the colorless epidermal cells after a short time. Plants growing out of doors show this reddish color, while those grown in the greenhouse do not. Leaves subjected to alkaline atmosphere show exactly the same red color development in the colorless epidermal cells. These observations would indicate that there is a decrease in acidity of the epidermal cells due to light which does not occur in the rest of the leaf. Similar changes might occur in the guard cells.

There are three theories which could be advanced to account for this decrease in acidity in the guard cells.

a. The decrease may be due to the accumulation of organic acids in darkness which are further oxidized to CO_2 and H_2O in the presence of sunlight. Such changes are known to occur in cacti. This theory would fail to account for the closure of stomata on wilted leaves in sunlight.

b. It may be due to some rearrangement of the components of protoplasm in light where acids are decreased.

c. It may be due to the accumulation of CO_2 from respiration which would be used in photosynthesis in sunlight, but not in darkness. Any cell low in buffer action would be changed considerably by its accumulation.

Micro-chemical tests for minerals in the guard cells showed negative results, but this is not conclusive proof of their absence because of the difficulty of making the test on such small cells. Low mineral content would mean low buffer action. More localized tests might definitely prove this point. The explanation of the behavior in light on permanent wilting could be explained on the theory of low buffer action by dilution or concentration of the H-ions due to loss of water by evaporation.

V. SUMMARY.

Measurements of the osmotic values of the different cells of the leaf were made by the plasmolytic method and by the depression of the freezing point. The guard cells increase in value in light, but no such variation in the other cells of the leaf were found.

Micro-chemical tests of the starch and sugar content of the guard cells and the starch content of the mesophyll cells were made. The increase in the osmotic value of the guard cells was correlated with a change of the starch to sugar in the guard cells.

The effect of non-toxic buffer solutions adjusted to known pH values was studied. It was found possible to open or close the stomata at any time either in light or in darkness by placing the epidermis in the proper pH value of the buffer solution. A theory is advanced that changes in acidity account for the change of starch to sugar in the guard cells. The probable causes of this change in acidity are considered, but more experimental data are necessary to prove the exact nature of this change.

Conclusions.

The data presented in this paper seem sufficient to warrant the following conclusions concerning the physiology of the stomata of patience dock:

1. The stomata of patience dock close completely at night and check the loss of water from the intercellular spaces of the leaf.

2. The opening and complete closure of the stomata is the principal cause of the periodicity of transpiration from the leaves.

3. When open the stomata modify the rate of water lost from the intercellular spaces of the leaf in proportion to changes in their perimeters, not to changes in their areas.

4. Sunlight is the principal environmental factor concerned in the opening and closing of the stomata, while the amount of water in the leaves and the acidity of the guard cells are the two internal conditions directly concerned with stomatal movements.

5. The guard cells of patience dock contain green plastids which are structurally, physiologically, and genetically different from the chloroplasts of the mesophyll cells.

6. The starch to sugar change in the guard cells is a reversible reaction which goes in either direction, depending on the changes in acidity of the guard cells.

7. The series of changes which results in the opening of the stomata is as follows:

(a) In the morning light decreases the acidity of the guard cells.

(b) This decrease in acidity makes conditions more favorable for the hydrolytic action of diastase.

(c) The diastase in the guard cells changes the starch to sugar.

(d) The formation of sugar results in an increase in the osmotic value of the guard cells.

(e) Water enters the guard cells from the epidermal cells which do not change in osmotic value and causes them to swell.

(f) The swelling of the guard cells causes the pore to open because the thickened cell wall around the pore stretches less than the thinner walls of the cell.

8. This series of changes does not take place if the leaves are wilted when light first falls on them. If wilting occurs after the stomata are open the reaction is reversed, so that the sugar changes to starch and the stomata close even in full sunlight.

9. In turgid leaves the stomata remain open until evening and then close by a reversal of each change which causes them to open.

10. By artificially changing the acidity of the guard cells in non-toxic buffer solutions the stomata can be opened or closed either in light or in darkness.

LITERATURE CITED.

1. Brown, H. T. and Escombe, F.
Static diffusion of gases and liquids in relation to the assimilation of carbon and translocation in plants. Ann. Bot. 14: 537–542. 1900.

2. Clark, W. M.
The determination of H-ion concentration. 3rd ed. Williams & Wilkins Co. 1925.

3. Darwin, F.
Observations on stomata. Phil. Trans. Roy. Soc. Lond. B. 190: 531–621. 1898.

4. Eckerson, Sophia H.
The number and size of stomata. Bot. Gaz. 46: 221–224. 1908.

5. Gray, J., and Pierce, G. G.
The influence of light upon the action of stomata and its relation to transpiration of certain grains. Am. Jour. Bot. 6: 131–155. 1919.

6. Iljin, W. S.
Die Regulierung der Spaltoffnungen in Zummenhang mit der Veranderung des osmotischen Druckes. Beihefte. Bot. Centbl. I 32. I. 15–35. 1914.

7. Iljin, W. S.
Die Permeabilitat des Plasmas fur Salze und die Analomosis. Studies Plant Physiol. Lab. Charles Univ. Prague 1: 97–119. 1923.

8. Knight, R. C.
Further observations on the transpiration, stomata, leaf water content, and wilting of Plants. Ann. Bot. 36: 361–383. 1922.

9. Knudson, L. and Ginsburg, S.
Suggestions with respect to the measurement of osmotic pressure. Am. Jour. Bot. VIII. No. 3. March. 164–171. 1921.

10. Laidlow, C. G. P., and Knight, R. C.
A description of the recording proometer and a note on stomatal behavior during wilting. Ann. Bot. 30: 47–56; 57–76. 1916.

11. Livingston, B. E., and Brown, W. H.
Relation of the daily march of transpiration to variations in the water content of foliage leaves. Bot. Gaz. 53: 309–330. 1912.

12. Lloyd, F. E.
The physiology of stomata. Carnegie Inst. Wash. Pub. 82. Washington. 1908.

13. Lloyd, F. E.
Leaf water and stomatal movements in gossypium and a method of direct visual observation of stomata in situ. Bull. Torey Bot. Club. 40: 1–26. 1913.

14. Loftfield, J. U. G.
The behavior of stomata. Carnegie Inst. Wash. Pub. 314. Washington. 1921.

15. Pool, Venus W., and McKay, M. B.
Relation of stomatal movement to infection by cercospora beticola. Jour. Agr. Research. V. No. 22. 1011–1038. 1916.

16. Renner, O.
Beitrage zur Physik der Transpiration. Flora 100: 451–547. 1910.

17. Renner, O.
Zur Physik der Transpiration. Ber. Deutschen Bot. Gesel. 29: 125–132. 1911.

18. Small, James.
A Theory of Geotropism. The New Phytologist 29: 49–63, 1920.

19. Thomas, Nesta, and Ferguson, A.
On evaporation from a circular water surface. Phil. Mag. 34: 308–321. 1917

20. Weber, Friedl.
Enzymatische Regulation der Spaltoffnungsberwegung. Naturwissenschaften 11: 309–316. 1923.

21. Wiggins, R. G.
Variations in the osmotic concentrations of the guard cells during the opening and closing of the stomata. Am. Jour. Bot. VIII. 30–41. 1921.

THE FIRST THREE LARVAL STAGES OF HEXAGENIA BILINEATA SAY.

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INTRODUCTION.

Although the study of the Ephemeridæ was begun by J. Swammerdam in the seventeenth century, our knowledge of the developmental stages through which these insects pass from the time they leave the egg until they appear on land as sub-imagos is still very limited. Quite a number of papers have appeared dealing with the morphology, embryology, taxonomy, biology, and the distribution of Ephemeridæ, but very little has been done towards working out the different stages in their post-embryonic development. The first work of this nature was done by Lubbock (1863–66) on the development of *Chloen dimiditum*. This was followed by the work of N. and E. Joly (1878) on *Paligenia virgo* and by that of Vayssiere (1882) on *Heptagenia longicauda*. More recently Murphey ('21) and Gros ('23) have worked out the post-embryonic development of *Baetis posticatus* and *Ecdyonurus forcipula* respectively.

THE MATERIAL.

The material on which the study of the first three larval stages of *Hexagenia bilineata* has been based was obtained at the Ohio State University Lake Laboratory at Put-in-Bay, Ohio. The larvæ were hatched in the laboratory from eggs that had been fertilized in normal salt solution and then incubated in lake water. The time required for incubation was nine days; this is one day less than the shortest period of incubation that I observed during the summer of '24 in the same laboratory. Clemens ('13) states that the time required for incubation is forty days. The length of time required for hatching varies perhaps with the individual eggs as well as with the temperature. Under identical conditions eggs from the same female fertilized by spermatozoa from the same male differed in the length of time required for incubation

from ten to twenty-four days. A low temperature would, I think, prolong the period required for incubation, for Murphey ('21) has shown that if in the development of *Baetis posticatus* the average monthly temperature is reduced from 62.4° to 45° the length of the aquatic existence is increased by fifty percent.

EGGS.

The female deposits on an average about 1500 eggs. Due, perhaps, to the crowded conditions in the abdomen of the female, the eggs are quite variable in shape; some are long ovals, some are elongate with square ends, again others have concave sides, etc. The eggs are whitish in appearance and measure about .27 mm. in length and .17 mm. in width. The chorion is rather heavy and is surfaced with regular hexagonal figuring. When hatching, the shell splits lengthwise to allow the emergence of the larvæ. The young larvæ become quite active soon after crawling out of the shell.

Because of the transparence of the body-wall, the alimentary canal—filled with yolk globules—can readily be seen, In the head it is a narrow tube, but it widens out abruptly in the prothorax and from there on it gradually decreases in width until the beginning of the hind-gut in the eighth abdominal segment. The beginning of the hind-gut is marked by the origin of the Malpighian tubules and the presence of the pyloric valve. The hind-gut is a short, straight tube. The Malpighian tubules, two in number, extend anteriorly along the ventral abdominal wall until they reach the metathorax, where they are bent dorsally and then extend posteriorly for a distance of several segments.

The dorsal vessel is also seen quite readily and one can see the blood corpuscles enter it and watch their passage from one chamber to the other.

STAGE I.

The newly hatched larvæ (Fig. 1) measure about .88 mm. in length, not including the antennæ and the caudal filaments. The head is almost quadrangular in shape except that the anterior border is more or less rounded off. From the anterior border of the head between the antennæ arises a small projection, (the clypeo-cephalic prolongement of Lestage). The head is .16 mm. wide and .14 mm. long. It is almost as wide

as the prothorax and rather wider than the meso- or the metathorax. The head bears five ocelli; an anterior pair, a posterior pair, and one unpaired median ocellus at the base of the clypeocephalic prolongement. All are approximately equal in size and circular in outline.

The antennæ (Fig. 5) .28 mm. in length, are composed of five segments of which the first is the shortest and of uniform thickness throughout, the second is rather longer than the first and increases in thickness towards the apex. All the three remaining segments are approximately equal in length and each about twice as long as the second. The second and third segments each bear one bristle near the apex, the fourth and fifth each has a pair of bristles in the same relative position.

The thorax measures .26 mm. in length and its three divisions show very plainly. The prothorax is slightly wider, as well as longer than either the meso- or the metathorax.

The abdomen measures .48 mm. in length and is composed of ten segments. Each of these is very distinct except the first one, which is so intimately joined to the metathorax that it is rather difficult to make out the presence of ten segments. The segments decrease in width, but increase in length from the first to the tenth. The length of the last four segments is half that of the entire abdomen.

The last abdominal segment bears three caudal filaments, (Fig. 6). Each of these is .32 mm. in length and composed of four segments of which the first three are approximately equal in length and the three together are slightly longer than the fourth. The first and second each has a finely toothed ridge near its distal end. The latter also bears one bristle near the apex. The fourth segment tapers down to a point and bears one terminal bristle. In *Ephemera simulans* the last segment bears two terminal bristles.

The fact that the lateral and the median filaments are equally well developed in Stage I distinguishes *Hexagenia bilineata* from several other species that have been described. Lubbock (1866) found that in the first stage of *Chloen dimidiatum* the two lateral filaments were composed of nineteen segments each. The median filament appeared as a minute knob in the second stage and did not show segmentation until the larvæ reached the sixth stage. Vayassiere (1882) found that in *Heptagenia longicauda* the median filament is much shorter than the lateral ones. Gros ('23) found just the opposite

relation in *Ecdyonurus forcipula*. Murphey found that in *Baetis posticatus* the median filament does not appear until the fifth stage.

The three pairs of legs (Fig. 4) are all the same and are already well developed; serving as organs of locomotion. The coxa and the trochanter are very short and well defined. The femur is likewise well developed and its length approaches one-third the length of the entire leg. The tibia is almost as long as the femur, and it bears two bristles. The tarsus is about half as long as the trochanter and likewise bears one bristle. Each leg terminates in a long, heavy claw.

The mouth-parts are in the first stage already fairly well developed. The labrum (Fig. 14) is a simple quadrangular shield bearing on the dorsal side of each anterior angle a well developed bristle. The mandibles (Fig. 8) show two well developed canines on the outer anterior border and a well marked molar surface on the inner edge. The maxilla (Fig. 7) bear a number of strong bristles on their free ends and show a slight development of the palpi. The labium (Fig. 9) as viewed from the ventral side appears like six lobular projections arising from a rectangular base. The two lateral lobules, destined to become the labial palpi, are a little longer than the remaining lobules which form the ligula, and each bears two small bristles, one near and one at the distal end.

This first stage, which lasts about four days, showed as far as I could discover, no trace of either trachea or gills.

STAGE II.

Specimens of this stage measure about (length not constant) .96 mm. in length. The antennæ have increased by the addition of a sixth segment and now measure .30 mm. in length. The caudal filaments have added one segment and are .36 mm. in length. The legs bear two additional bristles on the tibia and one on the femur. The ocelli show no modifications. The front of the head is modified by a decided increase in the clypeo-cephalic prolongement. However, the most important change has taken place in the abdomen. Along the posterior angles of segments 2 to 7 the gills (Fig. 2) have made their first appearance as little evaginations of the body wall. Apparently the gills arise from the dorsal part of the segment. There seems to be considerable difference of opinion on the origin of

the gills in Ephemeridæ. It has generally been assumed that they are pleural or even ventral in their formation. B. Duerken ('07), however, maintains that they arise from the tergi, while Carl Borner ('09) has claimed that they are modified coxa. On segments eight to nine no gills appear, but a long hair arises in the positions occupied by the gills in the preceding six segments.

The simultaneous appearance of six pairs of rudimentary gills in the second stage is remarkable and it distinguishes *Hexagenia bilineata* from most other species in which the early stages have been studied. Vayssiere found that in the first stage of *Heptagenia longicauda* rudiments of gills appeared on segments four and five. In the second stage those of the third and sixth segments appeared well formed, whereas, those of the second segment were rudimentary. Not until the third stage were six pairs of gills visible. In *Ecdyonurus forcipula* Gros ('23) found that gills appeared on segments five and six in the second stage and on four and seven in the fourth stage. It was not until the sixth stage that six pairs of gills were present. In *Chloen dimidiatum* Lubbock found no traces of gills until the third stage, when they were present on segments two to six, those on the third and fourth showing the highest degree of development. Heymons ('96) gives a figure of a young larvæ of *Ephemera vulgata* that shows an equal development of gills on segments two to seven. He does not say what stage it is, but from its appearance and comparing it with my larvæ of *Ephemera simulans*, I conclude that it is not beyond the second stage. Murphey ('21) states that in *Baetus posticatus* "gills show as tiny out-pockets of body wall," but does not mention the number.

In my larvæ of *Hexagenia bilineata* of the second stage the gills are all in the same stage of development and there is yet no trace of the seventh pair. No traces of tracheæ are found in either the gills or the body cavity.

The mouth parts of the second stage differ but little from those of the first stage. The canines and molar surfaces of the mandibles (Fig. 10) as well as the bristles and the palpi of the maxilla (Fig. 11) have increased in size. The labial palpi (Fig. 12) are now definitely separated from the ligular portion of the labium and are composed of two or three segments. The hypopharynx (Fig. 13) was not studied in the first stage,

although it undoubtedly exists there. It is in this stage composed of one median lobe and two lateral lobes which partially overlap the median lobe.

This second stage, like the first stage, lasts about four days.

STAGE III.

The antennæ show no change except a small increase in length. The caudal filaments have added two segments. The legs bear six hairs on the tibia and three on the femur.

There are still only six pairs of gills, but they are much larger (Fig. 14) and have become irregular in outline. The division between the gills and the abdominal segments is now much more definite and partakes of the nature of a joint. The gills move freely in an undulating manner.

In this stage the tracheal system makes its first appearance. Two longitudinal trunks, more lateral than median, extend the entire length of the body. Branches extend into the antennæ, towards the eyes, and towards the mouth parts. Branches extend also into the legs and to the muscles of the thorax. In the abdomen, branches segmental in their arrangement, are given off to the abdominal organs of the first eight segments and to the gills on segments two to seven. In the tenth abdominal segment each longitudinal trunk gives off a branch to the corresponding lateral caudal filament. I have been unable to establish definitely the origin of the tracheal branch of the median filament, but, presumably, it is similar to that in the damsel-fly, i. e., the two longitudinal trunks, after giving off the branches to the lateral filament, join and extend into the median filament.

I was unable to determine the length of the third stage as all my specimens died before the end of it. It lasts, however, at least as long as the previous stages.

In conclusion, I wish to express my gratitude to Professors C. H. Kennedy and Wm. S. Marshall for suggestions and criticisms.

LITERATURE CITED.

BORNER, C.
1908. Die Tracheenkiemen der Ephemeriden. Zool. Anz. XXXIII, p. 806.

CLEMENS, W. A.
1913. New Species and New Life Histories of Ephemeridæ or May-flies. Canad. Entom. XLV, p. 246.

DUERKEN, B.
1907. Die Tracheenkiemen Muskulatur der Ephemeriden unter Berucksichtigung der Morphologie des Insektenflügesl. Zeit. wiss. Zool. LXXXVII, p. 435.

GROS, A.
1923. Etudes Sur les Premiers Stades des Ephemeres du Jura francais. Ann. Biol. Lacustre XII, p. 49.

HEYMONS, R.
1896. Grundzuge der Entwicklung des Korberbaues von Odonaten und Ephemeriden. Abhandl. Kgl. preuss. Akad. Wissen zu Berlin.

JOLY, N. ET E'.
1878. Etudes sur les Metamorphoses et l'Embryogenie des Ephemerines et Especialement sur Celles de la Palingenia.

LESTAGE, J. A.
1917. Contribution a l'etude des larves des Ephemerines Palearctiques. Ann. Biol. Lacustra VIII, p. 213.

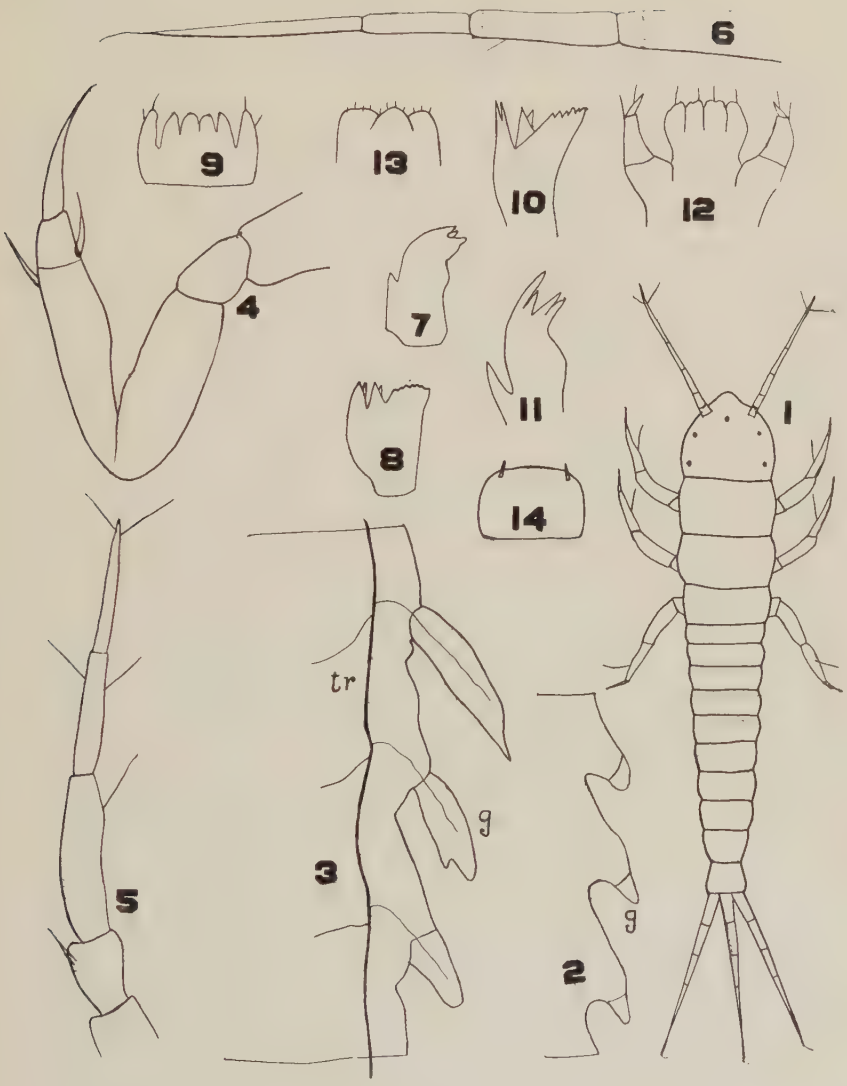
LUBBOCK, JOHN.
1866. On the Development of *Chloen* (Ephemera) *dimiditum*. Trans. Linn. Soc. XXIV, p. 61; XXV, p. 477.

MURPHEY, HELEN E.
1922. Notes on the Biology of May-flies of the genus Baetis. Bull. Lloyd Library No. 22, Entom. Ser. No. 2.

EXPLANATION OF PLATE.

Fig. 1. Larva of the first stage.
Fig. 2. Right half of abdominal segments 2–4 of the second stage showing beginning of gills.
Fig. 3. Right half of abdominal segments 2–4 of the third stage showing further development of the gills and the presence of trachea.
Fig. 4. Dorsal view of a leg of stage 1.
Fig. 5. Antenna of stage 1.
Fig. 6. Caudal filament of stage 1.
Fig. 7. Dorsal view of maxilla, stage 1.
Fig. 8. Dorsal view of mandible, stage 1.
Fig. 9. Ventral view of labium, stage 1.
Fig. 10. Dorsal view of mandible, stage 2.
Fig. 11. Dorsal view of maxilla, stage 2.
Fig. 12. Ventral view of labium, stage 2.
Fig. 13. Hypopharynx, stage 2.
Fig. 14. Dorsal view of labrum, stage 2.

Note: Fig. 1, X80; Figs. 2–14, X265. Abbreviations: g, gills; tr, trachea; lp, labial palpi.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SIAMESE TWINS OF ARISAEMA TRIPHYLLUM OF OPPOSITE SEX EXPERIMENTALLY INDUCED.*

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In an article published several years ago, the writer† described a number of dichotomous twins of *Arisaema triphyllum* and *A. dracontium*, all of which showed a very decided similarity of vegetative and reproductive characters and were also exactly alike in sexual expression. Because the reversibility of sex in Arisaema had been previously definitely established, the exact correspondence of the sexual nature of these as well as of other identical twins was thus known to give no weight to the often assumed hypothesis that the similarity of sex indicates a differential hereditary basis for maleness and femaleness. The conclusion was thus drawn that "identity of sex in duplicate twins can not be regarded as giving any conclusive evidence in support of the hypothesis that sex is determined by Mendelian factors." The following statement was also made: "Although so far the writer has no evidence that Arisaema twins placed in different environments would develop the opposite sexual states in a given season, yet, in view of the fact that any ordinary individual can be changed from season to season and that the pairs of twins have actually reversed their sex to the opposite state in agreement with a change in nutritive environment, it appears that such must be the case."

In the meantime Maekawa‡ has shown that the sexual condition of *Arisaema japonica* is essentially the same as in *A. triphyllum* and can be controlled by experimental means.

A small carpellate plant, transplanted to the garden, developed a dichotomous cleavage of the bud and thus became the subject of the experiments described below. This carpellate plant, along with others, was taken from the forest in 1922 and placed in an experimental plot. In transplanting, both the roots and leaves were injured to such an extent that in 1923 it

*Papers from the Department of Botany, The Ohio State University. No. 166.

†SCHAFFNER, JOHN H. The Sexual Nature of Vegetative or Dichotomous Twins of Arisæma. Ohio Jour. Sci. 22: 149-154. 1922.

‡MAEKAWA, TOKUJIRO. On the Phenomena of Sex Transition in Arisaema japonica Bl. Jour. Coll. Agr. Hokkaido Imp. Univ. 13: 217-305. 1924.

produced only a vegetative shoot. But it now showed definite signs of twinning, having two similar leaves distinct from the very base. The leaf surface was somewhat reduced by cutting off the tips and the plant was kept in comparatively dry condition during the spring growing season.

In 1924, according to expectation, definite twin shoots were produced and both were pure staminate, according to the treatment given for the control of the sexual state. The two shoots were still united for the most part by the original corm. These identical twins were absolutely alike in size and vegetative and reproductive characters. The anthers were a conspicuous red. From appearances, it seemed probable that these Siamese twins would be united for another season, so a careful attempt was made so to control the food supply of the two growing buds that one would change to female while the other remained in the male state. The leaf surface of the one now staminate twin was much reduced while its brother was left intact with a normal leaf development. The twins were then treated with an abundance of water for some time, and carefully watched and cultivated so that differential physiological gradients might be established in the two forks of the corm and differential growth and food storage take place. Of course, it was not possible to apply any accurate quantitative treatment, since food could easily be transferred from one side to the other. It was merely a matter of judgment as to what should be done, gained from former experience in controlling the sex of Arisaema. The attempt was to develop just the proper balance that the one bud would just reach the condition to induce female determination and still keep the other one reduced sufficiently to continue it in the male state. It was, of course, recognized that too great a deprivation of food of the proper sort would cause a sterile shoot without inflorescence to develop.

In the spring of 1925, the results of the experiment were awaited with anxious anticipations of success or failure. For as intimated above, it was well known that success would depend on a very close margin in the control of the storage and transference of food in the two buds of the still united corm. But when the two "jacks" began to break out of their "pulpits," all proved to have been done properly. For the "Siamese twins" were one staminate and the other carpellate—male and female. The one treated for staminateness was staminate and the one treated for carpellateness was carpellate. The attempted

balance had been properly accomplished. The female condition was just intense enough to hold out to nearly the end of flower development on the carpellate spadix, maleness being only very slightly expressed at the very last stage. There were a small staminate flower and a vestigial flower at the top of the inflorescence. The staminate plant was pure in sexual expression and also according to expectation, was noticeably smaller in size than the carpellate twin. See the accompanying Figure 1. The staminate plant had the same characteristics as the

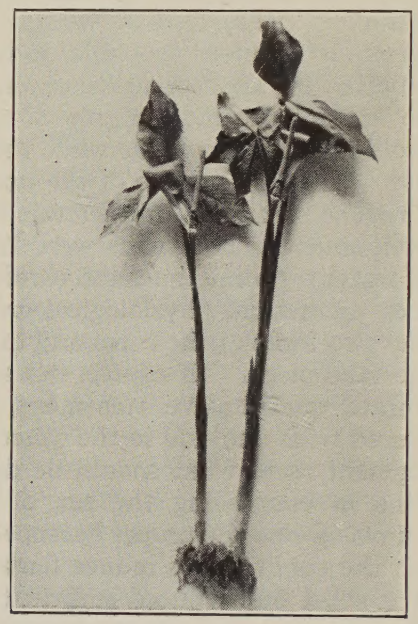

FIGURE 1.

Siamese twins of *Arisæma triphyllum*, one male, the other female. Note the difference in size between the staminate and carpellate plants. The spathes have been folded back so as to expose the spadixes.

staminate shoots of the previous season, with bright red anthers. The stigmas of the carpellate flowers were green, while the anthers of the small staminate flower, according to genetic expectation were typically red. Outside of the difference in size of the two plants there was also some difference in the robustness of the peduncles, that of the carpellate plant being the larger. The vegetative hereditary characters were exactly alike and the "jacks" or sterile tips of the spadixes were both

green as they had uniformly been at all previous developments. The sexual dimorphism of the two spadixes, however, appeared quite prominent because of the decided difference in color between the anthers and stigmas.

The twins were now dug up and examined carefully and were found still to have a considerable connecting bridge of living tissue between the two forks of the corm. Here then we have a case of plant Siamese twins, originally of the same sex, in which opposite sexual states were induced by a carefully estimated control of the food supply produced in the leaves together with a proper water and nutrient supply from the roots.

As stated in the previous paper and confirmed by further observations, the dichotomous twins of Arisaema are normally of the same type of sexual expression, whether staminate, carpellate, or some degree of moneciousness. It is only rarely that one finds twins, both those still united and those already separated but still growing side by side in the same intimate environment, that show some very slight difference in sexual development. Occasionally one can find carpellate twins for example in which one individual is pure carpellate and the other carpellate with one or two imperfect or perfect staminate flowers at the tip of the spadix.

It is established then, that sameness of sex in identical twins cannot be used as evidence for the hypothesis that sex is determined by heredity, nor for the supposition that identical twins have the same sex because they have the same heredity. Such a conclusion is unwarranted in any event, without additional support, because correspondence of phenomena does not establish a causal relation.

As is well known, in the nine-banded armadillo* nearly every female gives birth to quadruplets and all in a given set of quadruplets are of the same sex, either four females or four males. This indicates as in the case with identical twins of other higher animals that sex is determined in the egg before any cleavage takes place. Differentiation processes are set up which establish the given sexual state so that no reversal under ordinary conditions takes place. And this may be further intensified by the differential presence of allosomes having a pronounced effect on the established physiological

*See "The Physiology of Twinning," p. 100. H. H. Newman. The University of Chicago Press. 1923.

gradients. The continuity of the sexual states in the twinning process is, however, not different than its continuity in plants derived from cuttings in numerous higher species. Sexual conditions can become highly fixed through differentiation as well as can vegetative conditions. The primary cause of sex determination which starts a definite course of differentiation in the normal environment is undoubtedly the same in animals and plants, whether they have allosomes or not, since it is now known that both plants and animals with allosomes show sex reversal the same as those without such specialized chromosomes. There can be no question but that the sex of incipient twins, which have not passed through a differentiation cycle, is subject to reversal even more readily than that of mature or differentiated individuals. In the latter, dedifferentiation has been accomplished and the sex reversed in both plants and animals, by a proper manipulation of either the external or internal environment.

In conclusion, then, it can be stated that the sex of identical *Arisaema triphyllum* twins is normally the same, but that this does not at all imply that the sameness is due to a differential hereditary cause; since it is demonstrated that by a suitable manipulation of the environment, these identical twins, even after they have been developed as blooming plants and even while they are still connected by a considerable band of living tissue, can nevertheless be developed so as to be, simultaneously, the one a male and the other a female.

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